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Chimerism

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## INTRODUCTION

Vascularized composite allografts (VCA) are transplants containing multiple tissue types (including bone, muscle, skin, nerves and blood vessels), which offer patients restoration of function and form following severe, disabling and disfiguring injury or tissue loss, in circumstances where the results of conventional reconstructive surgery remain unsatisfactory. The high incidence of episodes of skin-targeted acute rejection, and the morbidity associated with current immunosuppression regimens, necessary throughout the life of the recipient to prevent rejection, remain significant areas in which improvement would enhance quality of life, improve the risk-benefit ratio of VCA and ultimately expand availability of these procedures to severely injured service men and women, and civilian victims of disabling and disfiguring trauma or disease. The objective of the VCA laboratory at the MGH is to develop a clinically-applicable strategy for the induction of immune tolerance of VCAs. The aim of the work supported by this award is to introduce and optimize a protocol for VCA tolerance based on the principle of delayed induction of mixed chimerism in a non-human primate (NHP) model. This approach, in contrast to protocols which have already reached clinical trials for kidney transplantation, permits induction of tolerance in the context of transplantation from deceased donors – a prerequisite for clinical application in VCA. Successful induction of tolerance for VCAs using this protocol in NHPs can be expected to lead to rapid translation into clinical trials.

## KEYWORDS

Vascularized composite allograft, vascularized composite allotransplantation, restorative transplantation, transplant tolerance, mixed chimerism, delayed induction of transplant tolerance, non-human primate model.

## OVERALL PROJECT SUMMARY

### i. Progress against Current Objectives

<u>Year</u>	<u>AIM</u>	<u>TASK</u>	<u>SUBTASK</u>	<u>MONTH(S)</u>	<u>% COMPLETE</u>
Year 1	AIM 1. To optimize the delayed tolerance induction protocol for vascularized composite allotransplantation in a non-human primate model.	(1.1) TASK 1. Investigate version 1 delayed tolerance induction protocol (DTIP) for upper extremity transplantation in nonhuman primates. (Months 0-18)	(1.1.1) SUBTASK 1. IACUC and ACURO review and approval. (Month 0-4)	Month 0-4	100%
			(1.1.2) SUBTASK 2. Order and take delivery of first cohort of non-human primates. (Month 5-6)	Month 5-6	100%
			(1.1.3) SUBTASK 3. Orthotopic upper extremity transplants on 4 months SIS (n=4). (Months 6-9)	Month 6-9	100%
			(1.1.4) SUBTASK 4. Delayed tolerance induction protocol, wean immunosuppression. (Months 10-13)	Month 10-13	100%
			(1.1.5) SUBTASK 5. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 10-18)	Month 10-18	100%

			(1.1.6) SUBTASK 6. Summarize preliminary data/progress on DTIP transplants for inclusion in year 1 report (Month 12)	Month 12	100%
Year 2	AIM 2. To investigate the effect of T memory cell inhibition and in-vivo T regulatory cell up regulation on the delayed induction of VCA tolerance.	(2.1) TASK 1. Investigate effect of Tmem inhibition on delayed induction of VCA tolerance (Months 12-24)	(2.1.1) SUBTASK 1. Heterotopic partial face transplants on 2 months SIS (n=4) (Months 12-15)	Month 12-15	0%
			(2.1.2) SUBTASK 2. Delayed tolerance induction protocol + CTLA4-Ig/rapamycin (Months 14-17)	Month 14-17	0%
			(2.1.3) SUBTASK 3. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 14-23)	Month 14-23	0%
			(2.1.4) SUBTASK 4. Summarize and report data on effect of Tmem inhibition on delayed induction of VCA tolerance for year 2 report (month 24)	Month 24	0%
		(2.2) TASK 2. Investigate effect of Treg up-regulation on delayed induction of VCA tolerance (Months 16-24)	(2.2.1) SUBTASK 1. Heterotopic partial face transplants on 2 months SIS (n=4) (Months 15-18)	Month 15-18	100%
			(2.2.2) SUBTASK 2. Delayed tolerance induction protocol + a-IL-6R (Months 17-20)	Month 17-20	100%
			(2.2.3) SUBTASK 3. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 17-24)	Month 17-24	100%
			(2.2.4) SUBTASK 4. Summarize and report data on effect of Treg upregulation on delayed induction of VCA tolerance for year 2 report (month 24)	Month 24	100%
Year 2-3	AIM 3. To investigate the effect of combined T memory cell inhibition and T regulatory cell up regulation on the delayed induction of VCA tolerance	(3.1) TASK 1. Investigate effect of combined Tmem inhibition and Treg up regulation on delayed induction of VCA tolerance (Months 22-36)	(3.1.2) SUBTASK 2. Heterotopic partial face transplants on 2 months SIS (n=4) (Months 25-28)	Month 25-28	100%
Year 3			(3.1.3) SUBTASK 3. DTIP with combined Tmem inhibition/Treg upregulation. (Months 24-30)	Month 24-30	0%
			(3.1.4) SUBTASK 4. Investigate durability of chimerism, VCA survival, frequency of complications (eg GvHD) and in vitro immune status (Months 24-36)	Month 24-36	0%
			(3.1.5) SUBTASK 5. Summarize and report data on effect of combined Tmem inhibition/Treg upregulation on delayed induction of VCA tolerance for year 3 report (month 36)	Month 36	0%

			(3.1.6) SUBTASK 6. Complete data analysis, prepare final (year 3) report, prepare manuscripts for submission	Month 36	75%
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ii. Methods

VCA Models

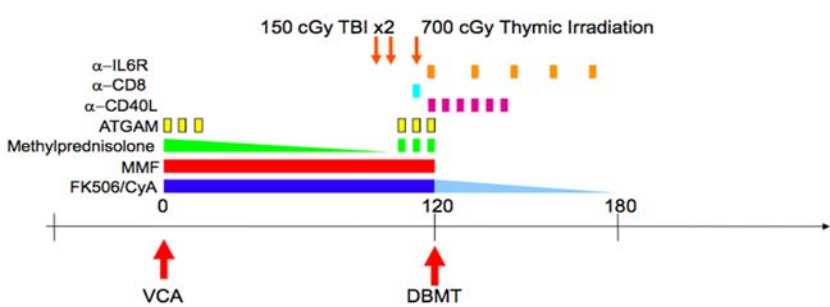
We performed two different surgical models on NHP during this study. First, orthotopic upper extremity as previously described (Leonard DA and al.). In front of the technical difficulties and the number of technical failures we decided to switch to a heterotopic partial face as described by Barth et al. Both models (Figure 1) consist of skin, muscle, inner gingival mucosa (only for face) and bone (including vascularized bone marrow), and allow investigation of the immune response to VCAs when transplanted under conventional immunosuppressive protocols.



**Figure 1:** (left): heterotopic partial Face VCAs  
(right) : orthotopic upper extremity VCA

Immunosuppression Regimen

VCA (vascularized composite allograft) from MHC-mismatched donors received induction with equine ATG (ATGAM; Pfizer, New York, NY; IV 50 mg/kg x 3 days on post-operative day (POD) 0, 1 and 2). Maintenance immunosuppression (delayed period was initially 4 months and then reduced to 2 months) consisted of FK506 (IM 0.1 mg/kg BID on POD 0 and 1, then adjusted to keep plasma levels between 20-30 ng/mL), MMF (CellCept; Genetech, San Francisco, CA; IV 300 mg on POD 0, then given parenterally QD mixed into the animal’s daily feed provided *ad libitum* with reduction to 100-200 mg QD by POD 14 and maintained to the end of the experiment), and methylprednisolone (Solu-Medrol; Pfizer, New York, NY; IV 40 mg on POD 0 and 1, followed by gradual taper over 14 days to IM 1g QD maintenance to the end of experiment) (Figure 2).



**Figure 2:** Immunosuppression regimen. Of note, the delayed period was initially 4 months and then reduced to 2 months.

## Rejection Monitoring and Histopathological Examination

All VCAs were monitored twice daily for the first 72 hours and once daily subsequently. Protocol skin biopsies of the VCA were performed using a standard 6 mm punch biopsy kit at approximately 30-day intervals. Clinical diagnosis of rejection (i.e. increased erythema, swelling, ulceration etc.) required biopsies of the affected area for histopathological confirmation and presumptive treatment with a steroid bolus and taper (SBT, similar to our maintenance regimen – starting from 40 mg before reduction to 1 mg/day over the ensuing 14 days). All skin biopsy samples were fixed in formalin and stained with hematoxylin and eosin (H&E). Histopathological examinations were performed by transplant pathologists blinded to the study. The severity of acute rejection was graded according to the 2007 Working Classification of Skin-containing Composite Tissue Allograft Pathology.

## Detection of Chimerism

Transplant pairs were pre-selected based on their differential reactivity to MHC class I antigens in NHPs using a specific antibody, H38. Chimerism (percentage of donor leukocytes) was assessed in peripheral blood via flow cytometry twice weekly during the first week, and once every fortnight thereafter. The following antibodies were used to assess chimerism: CD3, CD4, CD8 CD25, FoxP3, CD11b, CD2, CD154, CD95, CD45RO, mIgG (1,2,3). Samples were acquired on a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Inc., Ashland, OR).

## Mixed Lymphocyte Reaction Assay

The systemic immune status of recipient animals was assessed by proliferation dye based-mixed lymphocyte reaction (MLR) assays. Responder PBMCs from recipient animals were labeled with cell proliferation dye eFluor670 (eBioscience). Under vortex agitation, an eFluor670 solution was added to responder PBMCs and incubated at 37°C for 10 minutes. The responder cells were subsequently washed, counted and re-suspended at  $12 \times 10^6$  cells/mL (total 2 mL). Stimulator PBMCs from donor animals ( $2 \times 10^6$  cells/mL; total 2 mL) were irradiated at 2500 cGy. Labeled responder and stimulator cells were plated together in 96 well plates (Costar Corning; Lowell, MA) and incubated for 5 days at 37°C in 5% carbon dioxide and 100% humidity. Following this, cell proliferation of responder cells was analyzed with flow cytometry.

**Measurement of Donor-Reactive Antibodies** This was performed by flow cytometric analysis.

## Isolation and Characterization of Skin Resident Leukocytes

Punch biopsies taken from VCA skin. Epidermis and dermis were separated as protocol. The cells from the epidermis and dermis were stained with the following antibodies: CD3, CD4, CD8, CD25, CD45RO, CD45RA, CD207, mIgG3 and H38 and analyzed with flow cytometry.

### iii. Results

#### Study outcomes on initial cohort of animals on 4-Month Maintenance Immunosuppression

**Table 1: Recap of animals on 4-Month Maintenance immunosuppression**

Recipient	VCA	MHC-Mismatch	Complications	Average FK506	Allo-Antibody	Survival	Creatinine Glucose	Reason for Euthanasia
M1413	Hand	Full	<b>Banff II (POD 90)</b> Weight Loss	28.8 ng/mL (16.7 – 40.2)	No	> POD 120	0.6 – 0.9 53 – 157	Necrotizing fasciitis
M4213	Hand	Full	<b>Banff I (POD 30)</b>	25.7 ng/mL (5.7 – 48.7)	No	POD 51	1.1 – 1.4 47 – 175	VCA loss (Banff IV)
M6014	Face	Full	<b>Banff 0 (POD 19, 48, 78)</b> Weight Loss	23.3 ng/mL (9.0 – 38.9)	No	POD 107	0.6 – 1.1 74 – 157	<b>PTLD</b>
M6714	Face	Haplo-identical	Weight Loss	18.7 ng/mL (8.7 – 47.5)	No	POD 79	0.8 123	<b>PTLD</b>



**M1413** developed Banff II rejection on POD 97 (preceding FK506 level on POD 93 was 22.8 ng/mL) and resolved with SBT. He developed a necrotizing fasciitis after bone marrow transplantation (BMT) refractory to the wide specter of antibiotic and intensive care management.

**M4213** was transplanted successfully. The immediate post-operative appearance is shown in Figure 3 (A-C), note the presence of bright-red blood at the index finger tip in (C) which was elicited by needle stick, and demonstrates perfusion of the hand. Post-operative recovery went well, with no immediate complications noted. The hand remained well perfused on post-operative day (POD) 1, when radiographs confirmed good opposition of the radius and ulna, with no significant angulation (Figure 2 D, E).



**Figure 3. Immediate post-transplant appearance of transplanted hand (representative images recipient M4213).** A) Radial view, B) Volar view, C) Finger bleeding perfusion test, D) AP radiograph, E) Lateral radiograph.

On POD14 cellulitis with purulent discharge from the wound was diagnosed (Figure 4). The collection was drained, samples were sent for culture and antibiotic sensitivity screening and empirical antibiotic therapy started. This treatment resulted in rapid improvement, with relief of cellulitis, resolution of discharge with no further collection, and resolution of leukopenia over the next 7 days.



**Figure 4. Appearance of M4213 upper extremity transplant on post-operative day 14.** A) Volar view B) Radial view. Note erythema tracking across skin incision into recipient forearm, and swelling along ulnar border of distal recipient forearm in A.

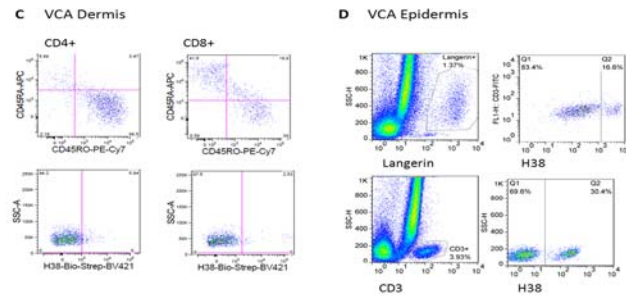
POD 30 erythema was noted (Figure 5A, B). Indeed, the biopsy was reported Banff I rejection. Per protocol, samples were collected for analysis of skin infiltrating leukocyte populations (figure 5C, D), peripheral blood T cell levels and anti-donor antibody production, and rescue therapy initiated with steroid bolus (methylprednisolone 40mg IV, to be followed by taper). Unfortunately, despite continued rescue therapy, skin rejection progressed and could not be reversed, necessitating removal of this animal from the study at POD51 with the primary end point of acute rejection prior to tolerance induction protocol.





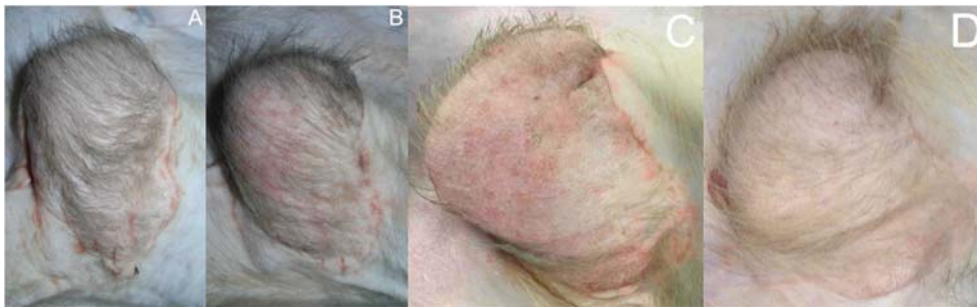
**Figure 5** A, B: Frank erythema of the hand witnessing of acute rejection episode

C, D : skin infiltrating leukocyte populations

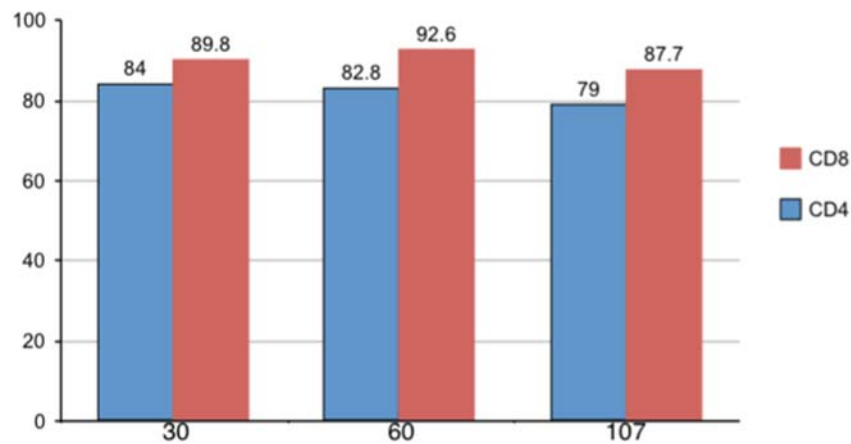


**M4413, M4313, M1214, M4113** were technical failure (hand model) -> We decided to change the surgical model after this series of failure.

**M6014** experienced 3 acute rejection episodes on POD 28, 48 and 60 (Figure 1A, B, C) that were all successfully treated and resolved with steroid pulses and at the time of euthanasia on POD 107, showed neither clinical nor histological signs of rejection in the VCA (Figure 1D). He was sacrificed due the development of PTLT.



**Figure 6. Clinical appearance of heterotopic partial face transplant in M6014.** Acute rejection episodes on (A) POD 28, (B) POD 48, (C) POD 60; (D) clinically viable VCA on POD 107 before euthanasia.



**Figure 7. Representative data from M6014.** (Top) Near complete turnover of resident T cells in VCA dermis to that of recipient-origin on flow cytometric analysis, (Bottom) percentage of CD4 and CD8 T cells in VCA dermis at POD 30, 60 and 107 (experimental end-point) demonstrating consistent numbers throughout.

**M6714** as the previous animal developed a PTLT POD 79 and was sacrificed due to the absence of curative treatment.

**M7114, M6914** were technical failures (face model)

## Study outcomes on initial cohort of animals on 2-Month Maintenance Immunosuppression

Due to PTLTD developing beyond two months on triple maintenance immunosuppression, we modified our study end point to POD 60. All remaining VCAs were also performed with the face model.

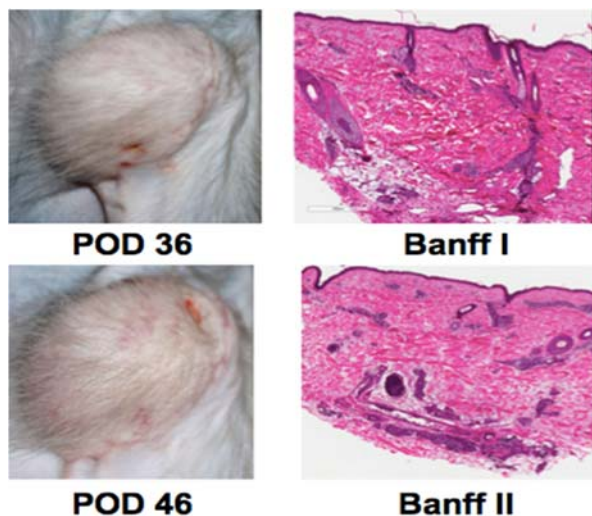
**Table 2: Recap of animals on 2-Month Maintenance immunosuppression**

Recipient	VCA	MHC-Mismatch	Rejection Episodes	Allo-Antibody	Survival (Days post BMT)	Days off IS (stopped on POD90)	C4d / GV	Reason for Euthanasia
M6514	Face	Full	Banff I (POD 36) Banff II (POD 46)	No	81 (10)	–	+ / no ^	Sepsis
M4415	Face	Full	Banff II (POD 14)	No	76 (14)	–	– / no	PTLD
M4515	Face	Full	Banff I (POD 15)	No	115 (53)	25	– / no	PTLD
M3316	Face	Haplo-identical	Banff I (POD 27)	No	93 (32)	4	– / no	PTLD
M3516	Face	Haplo-identical	Banff I (POD 12)	No	100 (38)	10	– / (yes)*	Self-mutilation
M3815	Face	Haplo-identical	Banff II (POD 126, 172, 195)	No	224 (163)	36, 14	+ / yes	Chronic rejection

^ only achieved 17% of target BMT dose

\* Focal endothelialitis (suggestive of early GV)

**M6514:** Animal subsequently developed acute rejection on POD 36 (Banff I) and 46 (Banff II) (Figure 7). He was treated (after skin biopsy) according to the protocol with a course of steroids (IV 40 mg bolus x 2 days, followed by gradual taper to 1 mg/day maintenance after 14 days).

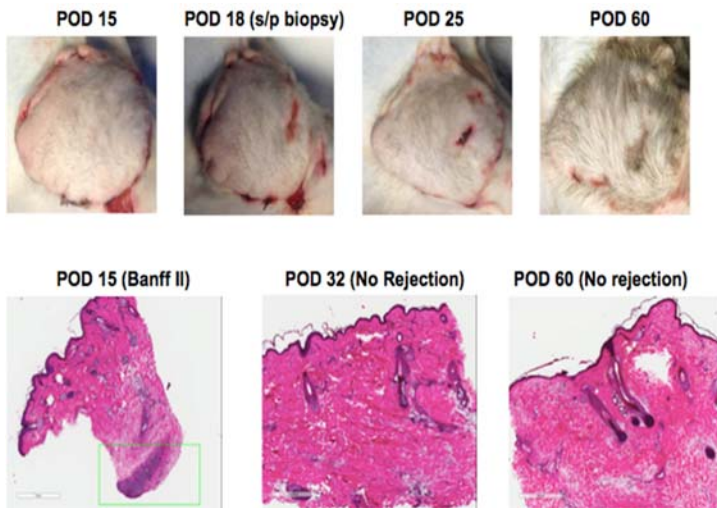


**Figure 7 M6514** acute rejection on POD 36 (Banff I) and 46 (Banff II)

This recipient animal did not receive enough donor bone marrow (BM) cells ( $50 \times 10^6$  cells/kg; target dose  $3 \times 10^8$  cells/kg) due probably to the storage process. At approximately 2 weeks after induction (i.e. BM infusion), the animal was found to have respiratory distress due likely, in part, to neutropenic sepsis from both the irradiation process and tocilizumab (known side effect). Corresponding WBC counts following conditioning were  $1.5 \rightarrow 1.5 \rightarrow 2.0 \rightarrow 0.6$ . Although fluoroquinolone-based antibiotic prophylaxis was given as per clinical practice (Simonsen et al, 2013), this was evidently inadequate coverage for NHPs. No evidence of mixed chimerism was detected but the VCA remained rejection-free at the experimental end point of POD 81.

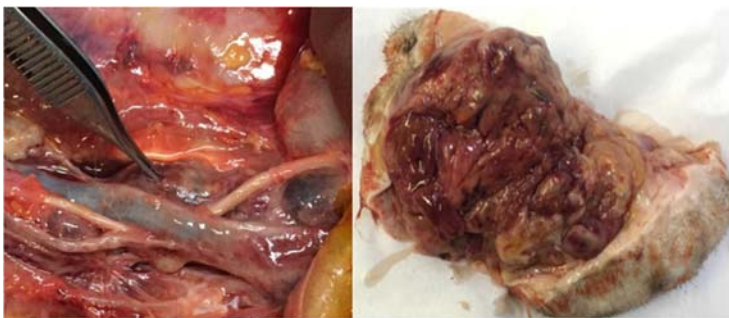
- In consultation with our transplant infectious disease experts at MGH, we revised our antibiotic prophylaxis regimen to IV vancomycin and IM cefepime for the next transplant surgery.

**M4415:** Animal subsequently developed acute rejection on POD 14 (Banff I) (Figure 8). In the same manner, he was treated (after skin biopsy) with a course of steroids (IV 40 mg bolus x 2 days, followed by gradual taper to 1 mg/day maintenance after 14 days).



**Figure 8.** Clinical course of M4415 and corresponding histology. Slight hint of rejection at approximately 2 and 7 o'clock position on POD 15; 2 o'clock position biopsied and revealed Banff II rejection in the deep dermis. Steroid bolus and subsequent taper was sufficient in achieving both clinical and histological resolution, and avoidance of recurrence and/or progression up to POD 60.

This recipient animal received the target dose of donor BM cells ( $3 \times 10^8$  cell/kg). Around 2 weeks after induction, there was a noticeable growth in size of the VCA itself. Despite conservative treatment with loop diuretics and omeprazole, a rectal mass was palpated and identified on ultrasound examination under sedation. In consultation with the veterinarian, a clinical diagnosis of PTLTD was made in view of our previous experiments and the animal had to be euthanized (Figure 8) as the growth of the mass was rapid. Again, the VCA remained rejection-free at the experimental end point of POD 76 but no evidence of mixed chimerism had developed. Final histopathology was indeed PTLTD with local invasion of the surrounding soft tissues and regional and distant metastases to the para-aortic and mesenteric lymph nodes.

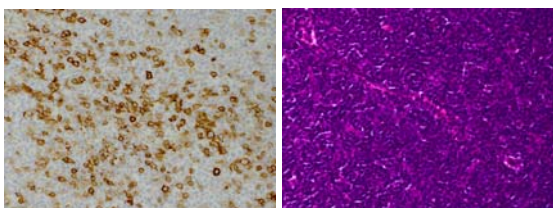


**Figure 9:** (left) Compression of the vena cava (right) and infiltration of the VCA by the tumor mass. Clinical signs: inferior extremity and scrotal edema, poor appetite and melanic stools.

**M4515:** Animal developed acute rejection on POD 15 (Banff I). Bolus of steroid was administrated accordingly. This recipient animal received the target dose of donor BM cells and received all dosages of anti-CD8, 5c8 (co-stimulatory blockade), and tocilizumab. Infective complications were avoided with our new antibiotic prophylaxis regimen of vancomycin and cefepime. This animal was successfully weaned off immunosuppression and was completely off for 25 days before it also developed PTLTD (Figure 10) and had to be euthanized on POD 115.



**Figure 10:** (up) tumor mass intra colon provoking intestinal bleeding. (down): histology showing proliferation of B cells (CD20+). Diagnostic of PTLTD





Final histology at experimental end-point revealed Banff I rejection although there were no clinical signs of rejection. Similarly, *in vitro* analysis failed to detect any evidence of mixed chimerism throughout all time points following donor BM infusion. Of note, skin leukocyte analysis at POD 90 showed that in the epidermis, 93.7% of Langerhans cells and 96.5% of CD3+ cells were recipient-derived; in the dermis, 99.9% of CD4+ cells and 98.6% of CD8+ cells were recipient-derived.

**M3316:** Animal developed Banff 1 rejection on POD 27, solved after bolus of steroid. This recipient animal received the target dose of donor BM cells and was progressing according to the protocol. Unfortunately, around 4 weeks after induction, there was a noticeable growth in size of the VCA itself. PTLD was diagnosed and we sacrificed him on POD 93. VCA was rejection free.

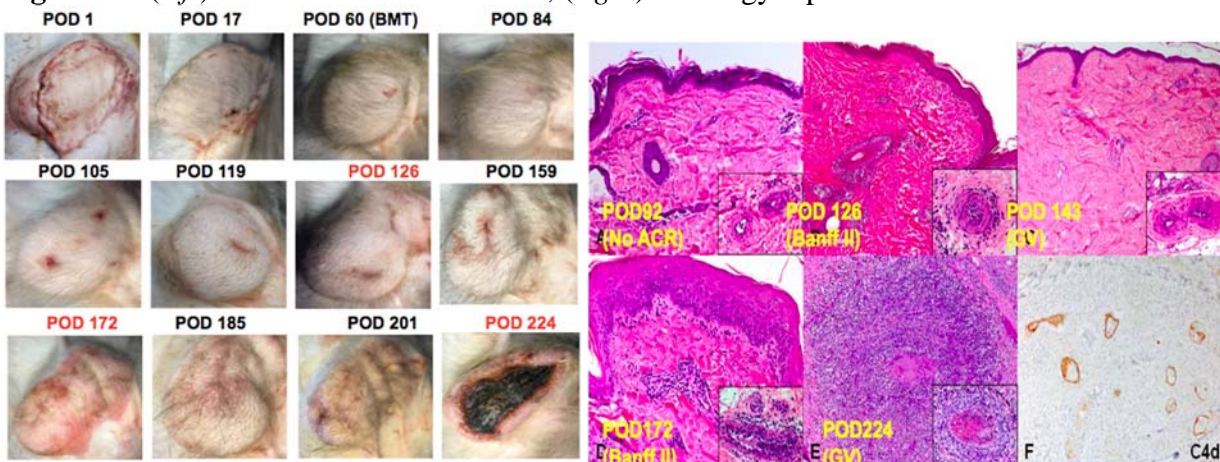
**M3516:** M3516 developed Banff I rejection on POD 12 when systemic levels of FK506 were 15.1 (target 20-30) ng/mL and the clinical appearance of the VCA was suspicious for rejection, and resolved with re-establishment of FK506 levels within our target range. This recipient animal received the target dose of donor BM cells and was progressing according to the protocol. Animal bit his VCA, we got back immediately to the OR for skin closure. Day 1 he recurred and we decided to sacrifice him.

**Figure 11** M3516 bit his VCA.

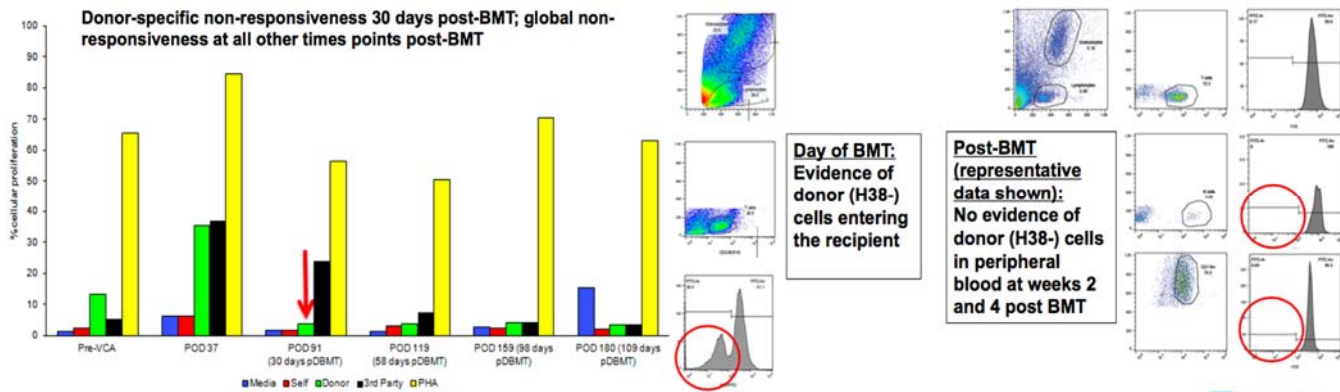


**M3815** remained rejection-free during the 2-months delay period and completed tolerance induction successfully with no adverse events. Following the weaning of immunosuppression, all medications were withdrawn for a total of 36 days before clinical rejection developed on POD 126, which was confirmed as Banff II on histology. In view of the possibility of a waxing and waning course before tolerance was fully established, we decided to treat the rejection episode by reinstating FK506, and a steroid bolus and subsequent taper (as per our protocol for treating rejection). The VCA was salvaged and on the follow-up biopsy on POD 143, both clinical and histological resolutions were achieved. We then withdrew immunosuppression again but this time, only about 2 weeks had lapsed before rejection recurred on POD 172 (Banff II). Again, we treated with FK506 and steroids but on further biopsy on POD 194, rejection persisted at Banff II. After consultation with the veterinarian, we decided not to treat this animal further and to let the VCA reject completely before euthanizing on POD 224.

**Figure 12.** (left) Clinical course of M3815, (right) histology reported.



In vitro, MLR showed POD 91 hypo responsiveness of donor party (Figure 13 (left)). We do not explain the general hypo responsiveness POD 159 and 180.

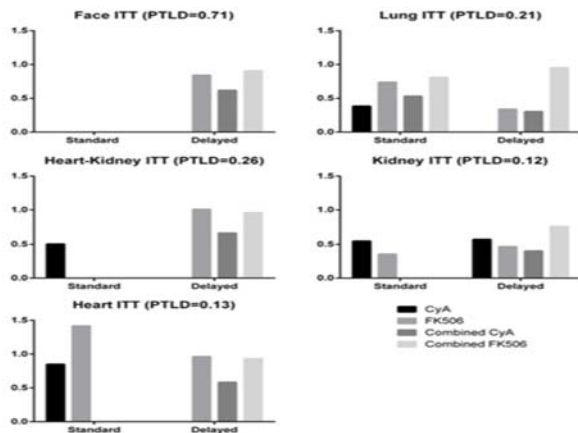


**Figure 13.** (right) Evidence of donor-origin (H38-) cells within the circulation after infusion of donor bone marrow cells. Gating from left to right. (left) MLR

#### iv. Discussion

##### Development of PTLD After Bone Marrow Infusion

The high incidence of PTLD (5/10, 50%) is concerning in this study. Previous studies in the field have attributed the high incidence of PTLD in NHP studies of VCA to simian lymphocryptovirus (LCV) which is genomically equivalent to human EBV (Barth et al, 2009). Unfortunately, testing of LCV status pre-transplant is not widely available and would presumably add on further direct experimental costs (Blossom 2007). It appeared that the risk of PTLD increase for vascularized composite allotransplant (face) (Figure 14). Analysis of a terminal tissue samples from M3316 demonstrated PTLD of recipient-origin (H38- by chimerism FACS), CD20+ B cells. Future studies ought to consider the use of rituximab for B cell depletion, or to replace ATGAM (largely T cell depletion) with alemtuzumab (depletes both T and B cells) (Kirk et al.). The usage of alemtuzumab for induction instead of ATGAM might be preferable as it is associated with a 71% increased risk of subsequent development of PTLD (Cherikh et al.). Implementation of this change would however, require switching from Mauritian to Indonesian strain NHPs due to the propensity of Mauritian strain macaques to develop hemorrhage following alemtuzumab administration (van der Windt et al.). In our protocol, we inject ganciclovir as prophylaxis even if it actively blocks lytic EBV replication *in vitro* through inhibition of the late phase lytic replication. However, neither agent have any effect on EBV in its latent state or on the proliferation of EBV-transformed B cells.



**Figure 14:** High incidence of PTLD for face to compare solid organ transplantation. We note a correlation between the level of FK-506 and the risk of PTLD. We observed a slight correlation between weight and PTLD incidence: Weight is correlated to age.

### **Failure to Develop Mixed Chimerism and Development of Chronic Rejection**

Our laboratory has previously shown that stable mixed chimerism is required for long-term tolerance of VCA in a swine model across single haplotype full MHC mismatch barriers (i.e. haploidentical recipients) (Leonard et al, 2014). Using the same swine model, we have also shown that the alternative hypothesis of transient mixed chimerism does not allow tolerance of VCAs (Leto Barone et al, 2015). Therefore, the failure to develop stable mixed chimerism with resulting rejection in our NHP study is not unexpected. The infusion of donor BM cells would technically be equivalent to transient mixed chimerism because we were able to detect the presence of these cells during the time of BM infusion. However, the immunomodulatory effect of donor BM cells was not long lasting and most likely, resulted in rejection once its effect had worn off. Despite the incidence of rejection episodes ranging from 1 to 3 in this study, no evidence of allo-antibody formation was detected in any of the study subjects. After necropsy, no thymuses in NHP were found (Figure 15). Maybe it is due to the age of NHPs (5-6 years in average) or the effect of the thymic irradiation responsible of its disappearance supposedly. This feature may limit clinical application of VCA tolerance to pediatric patients with functioning thymus. This suggests that age-related thymic involution may negate successful mixed chimerism-based tolerance induction strategies because of the failure to induce both central and peripheral tolerance.



**Figure 15:** No thymus was identified during the necropsy

### **Chronic rejection**

The identification and development of chronic rejection in our NHP model is also timely with recent similar reports emerging from long-term follow-up of VCA patients (Morelon et al) showing the first case of necrosis on the face. The aggressiveness and velocity of rejection starting from deeper tissues mirrors the clinical experience and lends further support to the need for successful tolerance induction strategies. We published an abstract showing the absence of relationship between C4d and vasculopathy.

### **KEY RESEARCH ACCOMPLISHMENTS**

The following represent key accomplishments of this research during this reporting period:

- *Optimization of techniques for analysis of cutaneous immune system in non-human primates.*
  - o These techniques, previously developed for human skin and optimized by our group for research in porcine models of VCA, permit comparative analysis of skin immune responses in multiple research species and in humans, which will facilitate broad translation of findings from this work to clinical application.
- *Correlation of flow cytometric analysis of cutaneous immune system with gross and histologic evidence of acute rejection.*



- Although preliminary, correlation of gross and histological evidence of rejection with infiltration of dermis and epidermis by recipient-type T cells, the majority of which bear a memory phenotype, is encouraging. This technique permits more comprehensive analysis of the cellular populations mediating rejection than can be achieved by histological analysis, and it can be hoped this will yield important mechanistic insights as this work progresses.
- *Optimization of the delayed tolerance induction protocol in non-human primates.*
  - By reducing the delay period from the original 4 months to 2 months, we have reduced the number of acute rejection episodes and were able to avoid rejection completely in haploidentical recipients. None of the recipient animals were lost to PTLD during this delay period either.
- *Short-to-medium term immunosuppression-free survival.*
  - We have shown, for the first time, successful short-to-medium term withdrawal of immunosuppression in a clinically relevant NHP model of VCA. Successful engraftment strategies would likely be required to achieve successful tolerance although alternative strategies such as a local tolerance approach through intra-graft delivery of FK506 may have to be considered.
- *Optimization of the delayed tolerance induction protocol in non-human primates.*
  - By reducing the delay period from the original 4 months to 2 months, we are confident that the number of acute rejection episodes can be minimized or eliminated completely even. This is particularly pertinent in view of our previous experiments where acute rejection led to irreversible VCA loss. In addition, decreasing the delay period will also reduce the overall duration of exposure to high-dose immunosuppression and the attendant risks of related complications such as PTLD and cachexia which can lead to irreversible weight loss and premature termination of the experiment.
- *Optimization of the heterotopic partial face transplant procedure in non-human primates.*
  - Following the unfortunate loss of M6914 after recovering from anesthesia, we have implemented closer, invasive intra-operative monitoring of blood pressure through the placement of an intra-arterial line in the femoral artery. Aggressive peri-operative preparation in the form of irradiated whole blood from the exsanguinated donor and recipient-typed blood from other experimental animals has ensured that blood transfusions are readily available as necessary for intra-operative support.

## CONCLUSION

The induction of transplant tolerance for reconstructive transplantation would be of considerable benefit to civilian victims of disabling and disfiguring tissue loss, and of significant importance to military victims of upper extremity and/or craniofacial trauma. Currently, the necessity of life-long immunosuppression and regular medical monitoring would prevent recipients of restorative transplants (such as hand or face transplant) from returning to active duty, but a safe and effective protocol for induction of transplant tolerance holds the potential to fundamentally change this paradigm.

The development of acute rejection during the delayed period is likely the main factor preventing cells engraftment. Based on the literature, sensitization of the recipient against donor cells reduce the chance of these cells to engraft. Future strategies will be to change to day 0 BMT and adjust the treatment to avoid PTLD. Recently our laboratory induced tolerance on swine with an immunosuppressive regimen including BMT day 0 + tacrolimus + belatacept + anti IL6 receptor + steroid. We would like to scale up to NHPs.

## PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

### 1. Lay Press:

Nothing to report

### 2. Peer-Reviewed Scientific Journals:

- Ng ZY, Read C, Kurtz JM, Cetrulo CL Jr. Memory T cells in vascularized composite allotransplantation. *Vasc Compos Allotransplantation* 2015;2(4):75-79.
- Leonard DA, Harrison P, Albritton A, Shanmugarajah K, Mastroianni M, Lofgren S, Winter J, Kurtz JM, Cetrulo CL Jr. Upper Extremity Transplantation in Non-Human Primates: An Orthotopic Model for Translational Research. *Vascularized Composite Allotransplantation* 2015;2(1):17-25.
- Ng ZY, Lellouch AG, Rosales IA, Leonard DA, Powell H, Gama AR, Schol IM, Colvin RB, Kurtz JM, Cetrulo, Jr CL Delayed Induction of Tolerance to Vascularized Composite Allografts in Non-Human Primates: I, Analysis of Acute Skin Rejection While On Maintenance Immunosuppression (in preparation)
- Lellouch AG, Ng ZY, Schol IM, Rosales IA, Leonard DA, Powell H, Gama AR, Colvin RB, Kurtz JM, Cetrulo, Jr CL Delayed Induction of Tolerance to Vascularized Composite Allografts in Non-Human Primates: Immunomodulation with Bone Marrow Transplantation and Tocilizumab
- Schol IM, Ko DSC, Cetrulo CL Jr Genitourinary vascularized composite allotransplantation. *Curr Opin Organ Transplant*. 2017

### 3. Invited Articles:

- Cetrulo CL Jr, Ng ZY, Winograd JM, Eberlin KR. Current concepts in microsurgery: the advent of vascularized composite allotransplantation. *Clin Plast Surg* 2017
- Lellouch AG, Ng ZY, Kurtz JM, Cetrulo CL Jr. Mixed chimerism based regimens in VCA. *Curr Transpl Rep* 2016;3(4):390-4.

### 4. Abstracts:

- Leonard DA, Mallard C, Shanmugarajah K, Mastroianni M, Albritton A, Powell H, Kurtz JM, Cetrulo CL Jr. Development of an Orthotopic Upper-Extremity Vascularized Allotransplant Model in Non-Human Primates. Poster Presentation. Harvard Medical School Surgical Research Symposium. Boston, Massachusetts. May 2014.
- Leonard DA, Mallard C, Shanmugarajah K, Mastroianni M, Albritton A, Powell H, Kurtz JM, Cetrulo CL Jr. Development of an Orthotopic Upper-Extremity Vascularized Allotransplant Model in Non-Human Primates. *Oral Presentation. New England Society of Plastic & Reconstructive Surgeons*. Sebasco, Maine. June 2014.
- Leonard DA, Mallard C, Shanmugarajah K, Mastroianni M, Albritton A, Powell H, Kurtz JM, Cetrulo CL Jr. Development of an Orthotopic Upper-Extremity Vascularized Allotransplant Model in Non-Human Primates. *Poster Presentation. Military Health System Research Symposium*. Fort Lauderdale, Florida. August 2014.
- Leonard DA, Mallard C, Shanmugarajah K, Mastroianni M, Albritton A, Powell H, Kurtz JM, Cetrulo CL Jr. Development of an Orthotopic Upper-Extremity Vascularized Allotransplant Model in Non-Human Primates. *Poster Presentation. American Society for Reconstructive Transplantation Biennial Meeting*. Chicago, Illinois. November 2014.
- Ng ZY, Lellouch AG, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Jr. Immunomodulation in pediatric vascularized composite allotransplantation – preliminary results in a non-human primate model with tocilizumab. *Poster Presentation. 8<sup>th</sup> Annual MassGeneral Hospital for Children Research Day, Boston, MA; 29 March 2016*

- Ng ZY, Leonard DA, Defazio MW, Heroux ZW, Kurtz JM, Cetrulo CL Jr. Challenges of triple immunosuppression in non-human primate models of vascularized composite allotransplantation. *Oral Presentation. 61<sup>st</sup> Annual meeting of the Plastic Surgery Research Council, New York, NY; 21 May 2016*
- Ng ZY, Lellouch AG, Defazio MW, Powell H, Leonard DA, Heroux ZW, Cetrulo CL Jr, Kurtz JM. Analysis of acute skin rejection in non-human primate models of face and hand allotransplantation. *Oral Presentation. 57<sup>th</sup> Annual meeting of the New England Society of Plastic and Reconstructive Surgeons, Bretton Woods, NH; 11 June 2016 (Shortlisted for Joseph E. Murray, M.D. Award)*
- Ng ZY, Lellouch AG, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Jr. Towards tolerance of facial allotransplantation – preliminary results in a non-human primate model with tocilizumab. *Poster Presentation. Military Health Sciences Research Symposium, Kissimmee, FL; 17 August 2016*
- Ng ZY, Defazio MW, Powell H, Leonard DA, Heroux ZW, Lellouch AG, Cetrulo CL Jr, Kurtz JM. Analysis of acute skin rejection in non-human primate models of face and hand allotransplantation. *Oral Presentation. 26<sup>th</sup> International Congress of The Transplantation Society, Hong Kong; 23 August 2016*
- Lellouch AG, Ng ZY, Rosales IA, Colvin RB, Gama A, Schol IM, Geoghegan L, Kurtz JM, Cetrulo CL Jr. Delayed tolerance induction protocol for vascularized composite allografts in non-human primates: the immunomodulatory effect of donor bone marrow transplantation does not prevent the development of chronic rejection in the absence of durable mixed chimerism. *62<sup>nd</sup> Annual meeting of the Plastic Surgery Research Council, Durham, NC; 5 May 2017*
- Ng ZY, Lellouch AG, Defazio MW, Powell H, Leonard DA, Heroux ZW, Kurtz JM, Cetrulo CL Jr. Analysis of acute skin rejection in non-human primate models of face and hand allotransplantation (Shortlisted for Joseph E. Murray, M.D. Award). *57<sup>th</sup> Annual meeting of the New England Society of Plastic and Reconstructive Surgeons, Bretton Woods, NH; 11 June 2016*
- Ng ZY, Lellouch AG, Defazio MW, Heroux ZW, Shah JA, Kurtz JM, Cetrulo CL Jr. Immunomodulation in vascularized composite allotransplantation – preliminary results in a non-human primate model with tocilizumab. *American Society of Plastic Surgeons Annual meeting, Los Angeles, CA; 24 September 2016 (Awarded Outstanding Paper Presentation in Research & Technology Track)*
- Rosales IA, Defazio M, Foreman RK, Sachs DH, Cetrulo CL, Colvin RB, Leonard DA Systematic pathological component scores for skin-containing vascularized composite allografts *13th Congress of the International Society of Vascularized Composite Allotransplantation 2017*

## INVENTIONS, PATENTS AND LICENSES

Nothing to report.

## OTHER ACHIEVEMENTS

Current post-doctoral research fellows have received the following awards:

- Royal College of Physician and Surgeons of Glasgow College Traveling Fellowship (Ng ZY) Awarded 2016
- American Society for Surgery of the Hand Annual Meeting Scholarship (Ng ZY) Awarded 2016
- Assistance Publique des Hôpitaux de Paris (Lellouch AG) Awarded 2016 and 2017
- Master of Science degree (Lellouch AG)
- Medical Z Laboratory (Lellouch AG) Awarded 2016
- Ng ZY. Young Investigator Award, International Society of Vascularized Composite Allotransplantation 2017.
- Ng ZY. Speaker, Vascularized Composite Tissue Allotransplantation Workshop: "Latest Research into VCA Immune Tolerance." SingHealth Duke-NUS Surgical & Anaesthesia Congress 2017
- Ng ZY was also invited as a guest speaker at the 1<sup>st</sup> Face and Hand Transplant Update back in his home country of Singapore.

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- Leto Barone AA, Kurtz JM, Albritton A, et al. Effects of transient donor chimerism on rejection of MHC-mismatched vascularized composite allografts in swine. *Vasc Comp Allotransplantation* 2015;2(1):1-8
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- Barth RN, Bluebond-Langner R, Nam A, et al. Facial subunit composite tissue allografts in nonhuman primates: I. Technical and immunosuppressive requirements for prolonged graft survival. *Plast Reconstr Surg* 2009;123(2):493-501
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Leonard DA, Kurtz JM, Cetrulo CL Jr. Vascularized composite allotransplantation: towards tolerance and the importance of skin specific immunobiology. *Curr Opin Organ Transplant* 2013;18:645-51

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## ABBREVIATIONS

ATG	anti-thymocyte globulin (equine)
FK506	tacrolimus
GvHD	graft versus host disease
IM	intra-muscular
IV	intra-venous
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
NHP	non-human primate
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
POD	post-operative day
PTLD	post-transplant lymphoproliferative disorder
SBT	steroid bolus and taper
SOT	solid organ transplantation
VCA	vascularized composite allograft

## APPENDICES

None



# Tolerance in Nonhuman Primates by Delayed Mixed Chimerism

Log #: 120034P5

Award #: W81XWH-13-2-0062

PI: Curtis L. Cetrulo, Jr., M.D., FACS

Org: Massachusetts General Hospital

Award Amount: \$1,299,776



## Study/Product Aim(s)

SA1: To optimize the delayed tolerance induction protocol for vascularized composite allotransplantation in a non-human primate model

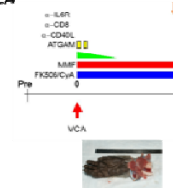
SA2: To investigate the effect of T memory cell inhibition and in-vivo T regulatory cell up regulation on the delayed induction of VCA tolerance

SA3: To investigate the effect of combined T memory cell inhibition and T regulatory cell up-regulation on the delayed induction of VCA tolerance

## Approach

Optimization of delayed tolerance induction protocol in preclinical NHP model of hand transplantation. Delayed tolerance has been successfully induced for organ transplantation in our center; in Aim 1 we will optimize this protocol for VCA. In aim 2 we will investigate tipping balance of negative memory cell and protective, regulatory cell function in favor of tolerance. In aim 3 we will combine these approaches and test in both hand and face transplant models.

SA1: Optimize delayed tolerance protocol for VCA



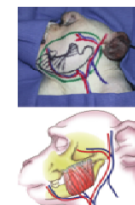
T memory cell inhibition

T regulatory cell up-regulation

SA2: Investigate effect of Tmem vs Treg balance on delayed VCA tolerance

SA3: Test DTIP + combined T mem inhibition/Treg up-regulation in NHP hand and face transplant

Future goal: Hand/Face transplant without long-term immunosuppression



Accomplishment: Development of optimal delayed tolerance induction protocol (DTIP) for hand and face transplantation. Successful implementation of this protocol might permit hand/face transplant without lifelong medication.

Activities	CY	13	14	15	16-17
1.1. Investigate version 1 DTIP for face and hand transplantation in NHPs					
2.1. Investigate effect of Tmem inhibition on hand and face transplants in NHPs on DTIP					
2.2 Investigate effect of Treg up-regulation on hand and face transplants in NHPs on DTIP					
3.1. Investigated combined Tmem inhibition/Treg up-regulation on hand and face transplant in NHPs					
<b>Estimated Budget (\$)</b>		28	389	630	253

## Goals/Milestones

### CY14 Goals

- Investigate delayed tolerance induction protocol for face and hand transplantation in NHPs
- Determine efficacy of basic DTIP for VCA in NHPs

### CY15 Goals

- Investigate effect of Tmem inhibition on DTIP for NHP VCAs
- Investigate effect of Treg up-regulation on DTIP for NHP VCAs

### CY16-17 Goals

- Test combined Tmem inhibition/Treg up-regulation on DTIP for VCAs
- Conclude data analysis, prepare reports and manuscripts

## Comments/Challenges/Issues/Concerns

- Treg up-regulation appears to promote short-medium term immunosuppression-free survival (2-5 weeks) of the VCA (n=3).
- Three recipients developed PTLD after receiving bone marrow
- In the absence of durable mixed chimerism, long-term survival resulted in the development of chronic rejection despite donor bone marrow infusion
- No chimerism detected after BMT in all our NHPs

## Budget Expenditure to Date

Projected Expenditure: \$1,299,776

Actual Expenditure: \$1,236,450

Updated: October 15<sup>th</sup>, 2017